THE EFFECT OF THE DIPHTHERIA TOXIN ON THE BLOOD AND HEMOPOIETIC ORGANS OF RABBITS

WITH PLATE

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Although many details have been added to the knowledge of diphtheria toxin since its discovery, still its effect on the blood is not fully understood.

Bouchut and Dubrisay[1] noted leukocytosis as usual in diphtheria, and later the former[2] found that the number of leukocytes increases with the severity of the disease and decreases on improvement. After a careful study of leukocytic reaction in animals and in clinical cases, Gabritchewsky[3] concluded that a progressive leukocytosis signifies a bad prognosis in diphtheria, and this is in accord with the results of Nicolas and Courmont,[4] Schlesinger,[5] and Morse,[6] but contrary to those of Besredka,[7] who observed that leukocytosis has no relation to the severity of the disease, but that a decrease in the number of polymorphonuclear leukocytes, together with the presence of "intermediate cells," means an unfavorable prognosis. Furthermore, his results showed that in the animals inoculated with a massive dose of diphtheria toxin the number of leukocytes after reaching its maximum gradually declined till the death of the animal. Ewing[8] stated that in fatal cases the number of leukocytes may be steadily increased or decreased, or there may be no leukocytosis. Arneth[9] found leukopenia in one fatal case, and also recorded the occurrence of myelocytes in two fatal cases. As to the presence of myelocytes in the blood of diphtheria patients, Engel[10] was the first to call attention to the fact that the number of myelocytes increases as the disease advances. He found from 3 to 11 % of myelocytes in the fatal cases.

Gabritchewsky[3] demonstrated necrosis of leukocytes in rabbits inoculated with diphtheria bacilli in the anterior chamber of the eye, the leukocytes appearing in large numbers at 8 hours and reaching its maximum at 24 hours after inoculation. Metchnikoff[3] observed the same phenomena and concluded that phagocytosis by leukocytes in diphtheria plays a minor part. Ewing[8] claimed that there is a marked leukocytic degeneration as evidenced by the presence of leukocytic shadows of Klein and deficiency in the granules and chromatin of polymorphonuclear leukocytes; the latter condition in one case

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1 Compt. rend. Acad. d. sc., 1877, 85, p. 158.
2 Gaz. d. hôp., 1879, 52, p. 153.
3 Ann de l'Inst. Pasteur, 1894, 8, p. 673.
4 Arch. de méd. expér., 1897, 9, p. 737.
5 Arch. f. Kinderheilk., 1896, 19, p. 378; 1900, 30, p. 233.
6 Boston Med. & Surg. Jour., 1895, 132, pp. 228, 252.
7 Ann. de l'Inst. Pasteur, 1898, 12, p. 305.
8 Clin. Path. of the Blood, 1903, p. 290.
9 Die neutrophilen weissen Blutkörperchen, 1904, p. 79.
10 Deutsch. med. Wchnschr., 1897, 23, pp. 118 and 137.

was associated with leukopenia. He also detected an increased acidophile tendency in the neutrophile granules. Welch and Flexner [11] noted karyorrhexis of leukocytes in the lungs and liver of their animals. Barbacci,[12] studying the changes of the spleen, lymph nodes and liver in diphtheria, found chromatin materials in the splenic pulp, which were supposed to be derived from degenerated leukocytes. He called diphtheria toxin karyolytic and said that it works not only on the fixed lymphatic elements, but also on the mobile the leukocytes of the blood. Schurmann [13] induced karyorrhexis of leukocytes in vitro by diphtheria toxin.

Councilmann, Mallory and Pearce [14] observed a hyperplasia of marrow as a usual occurrence in diphtheria, and in one case they found an area of hemorrhage and necrosis. Roger and Josus [14] studied the effect of diphtheria toxin and antitoxin on the marrow; they found that the former produces a proliferation of large and medium cells and the latter that of small cells. Trambusti [14] called attention to the fact that the reaction of marrow depends on the amount of toxin, large doses causing a paralytic action and a small one hyperplasia. Morse [9] described a hyperplasia of small cells with small round and deeply staining nuclei. Dickson [15] found vacuolation and karyorrhexis in marrow cells to be induced by diphtheria toxin.

The changes in the spleen and lymph nodes in diphtheria have been thoroughly described by various authors. However, the question of the large cells in the center of malpighian bodies still remains open. Bizzozero [12] was the first to describe the large cells with pale, round or oval nuclei that sometimes carry globular bodies within the cytoplasm. Oertel [16] emphasized their epithelial character. Ziegler [17] considered them as swollen cells of the reticulum and Ribbert [18] thought that they are derived from the endothelium. Councilmann, Mallory and Pearce [14] found these cells usually in the early stages of infection and regarded them as similar to the large cells in tubercle and as phagocytic. Waschkewitsch [19] claimed that they are degenerated leukocytes which have wandered into the malpighian bodies.

The general technic applied in this study is the same as described in my previous article [20] on the effect of hemolytic streptococci on the blood and hemopoietic organs of rabbits. The diphtheria toxin tested 0.012 c c MLD. The animals were inoculated intravenously with various amounts of diphtheria toxin in 1 c c of salt solution.

The following tables serve to illustrate the changes in the leukocytes in vivo and vitro.

[11] Bull. Johns Hopkins Hosp., 1896, 2, p. 107.
[12] Centralbl. f. Allg. Path., 1896, 7, p. 321.
[13] Ibid., 1910, 21, p. 337.
[14] Jour. Bost. Soc. Med. Sc., 1900, 5, p. 139.
[15] The Bone-Marrow, 1908.
[16] Die Pathologie der epidemischen Diphtheritis, München, 1887.
[17] Text-Book of Spec. Path. Anat. (Eng.), 1897, sec. 3, p. 110.
[18] Lehrbuch der Path. Anat., 1896.
[19] Virchows Arch., 1900, 159, p. 137.
[20] Jour. Infect. Dis., 1921, 29, p. 141.

Experiment 1.—A rabbit, weighing 3,004 gm., received 0.6 c c of diphtheria toxin. The animal appeared very sick for 2 hours after inoculation and died within 20 hours.

TABLE 1
FINDINGS IN EXPER. 1

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Before inoculation	12,000	29.5	1.5	0	67.5	1.5	0	0	0
½ hour after	10,000	28.5	3.0	0.5	67.5	0.5	0	0	0
1 hour after	9,000	70.5	1.0	3.0	24.5	1.0	0	0	0
2 hours after	6,550	69.5	2.5	0	26.0	2.0	0	0	32
4 hours after	3,550	59.0	4.5	0	35.5	1.0	0	0	45
6 hours after	5,020	12.0	0	0.5	80.0	2.5	0	5	46
16 hours after	4,850	24.5	3.5	0.5	61.0	2.5	0	8	62

Experiment 2.—A rabbit, weighing 2,750 gm., received 0.4 c c of diphtheria toxin and died within 24 hours.

TABLE 2
FINDINGS IN EXPER. 2

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Before inoculation	14,300	35.5	3.5	0.5	60.0	0.5	0	0	0
½ hour after	12,500	46.5	1.0	0.5	51.5	0.5	0	0	0
1 hour after	11,950	56.0	3.0	0	39.0	2.0	0	0	0
2 hours after	12,050	54.5	3.5	0	40.0	2.0	0	0	26
4 hours after	9,675	52.0	4.5	0	41.5	2.0	0	0	19
6 hours after	8,450	36.0	3.0	0.5	26.0	4.0	0	30.5	21
16 hours after	7,650	38.0	3.5	0	29.0	7.0	0	22.5	47

Experiment 3.—A rabbit, weighing 2,875 gm., received 0.1 c c of diphtheria toxin and also died within 24 hours.

TABLE 3
FINDINGS IN EXPER. 3

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Before inoculation	10,725	42.5	5.0	0	51.5	1.0	0	0	0
½ hour after	10,950	45.0	4.0	0	50.0	1.0	0	0	0
1 hour after	10,650	41.5	4.5	0	49.0	5.0	0	0	0
2 hours after	10,600	46.5	3.0	0	44.5	6.0	0	0	0
4 hours after	10,675	57.0	2.5	0	40.5	0	0	0	0
6 hours after	8,550	41.5	4.0	0	32.5	0	0	12	13
16 hours after	10,825	39.0	2.5	0	24.5	0	0	34	18

Experiment 4.—Rabbit A, weighing 2,415 gm., received 0.05 c c of diphtheria toxin and died about 30 hours after inoculation.

Rabbit B, weighing 2,600 gm., received 0.05 c c of diphtheria toxin; and died in about 48 hours.

TABLE 4
FINDINGS IN EXPER. 4

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Rabbit A:									
Before inoculation	12,925	36.5	4.5	0	54.0	5.0	0	0	0
½ hour after	12,850	40.0	3.0	1.0	52.0	4.0	0	0	0
1 hour after	13,250	38.5	3.5	1.0	53.5	3.5	0	0	0
2 hours after	12,950	39.0	3.5	0	54.0	3.5	0	0	0
4 hours after	13.000	34.5	2.0	0.5	59.0	4.0	0	0	0
6 hours after	9.025	45.5	3.5	0	47.0	4.0	0	0	0
16 hours after	12,050	46.0	3.0	1.0	48.0	2.0	0	0	0
20 hours after	13,750	49.5	4.5	0.5	39.0	6.5	0	0	0
24 hours after	41,800	32.5	1.5	0	61.0	5.0	0	0	23
Rabbit B:									
Before inoculation	12.025	26.0	1.0	0.5	72.0	0.5	0	0	0
½ hour after	12,450	31.0	2.5	0	64.0	2.5	0	0	0
1 hour after	11,950	30.0	3.0	0.5	66.0	0.5	0	0	0
2 hours after	9,675	45.5	5.0	0.5	49.0	0	0	0	0
4 hours aftre	10.950	48.0	1.5	1.5	49.0	0	0	0	0
6 hours after	12,000	52.0	3.0	0	37.0	8.0	0	0	0
8 hours after	14,250	58.5	4.0	0.5	29.0	8.0	0	0	0
24 hours after	53,750	69.0	0.5	0	30.5	0	0	0	32
30 hours after	34,500	51.5	2.5	0	18.5	2.5	0	25	46

Experiment 5.—A rabbit, weighing 2,456 gm., received 0.01 c c of diphtheria toxin and died on the fourth day after inoculation.

TABLE 5
FINDINGS IN EXPER. 5

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Before inoculation	14.425	37.5	2.0	0	60.0	0.5	0	0	0
½ hour after	9,975	31.5	1.5	0	66.0	1.0	0	0	0
1 hour after	8,125	37.5	3.0	1.0	56.5	2.0	0	0	0
2 hours after	9,675	33.0	3.0	0	64.0	0	0	0	0
4 hours after	8,225	41.0	3.0	0	54.5	1.5	0	0	0
6 hours after	12,175	42.0	0.5	0	56.5	1.0	0	0	0
24 hours after	12.650	50.0	3.5	0	46.5	0	0	0	0
30 hours after	14,450	56.0	2.5	0	39.5	2.0	0	0	0
48 hours after	10,675	61.5	3.0	0	34.0	1.5	0	0	0
54 hours after	10,625	59.5	3.0	0	32.5	5.0	0	0	0
72 hours after	24,375	38.5	4.0	0.5	34.5	9.5	0	13	0

Experiment 6.—Rabbit A, weighing 2,350 gm., was immunized with 1,500 units of diphtheria antitoxin 12 hours before receiving 0.1 c c of diphtheria toxin. The animal survived, but both hind legs were paralyzed on the fourth day of the experiment.

Rabbit B, weighing 2,650 gm., was immunized with 1,500 units of diphtheria antitoxin 12 hours before receiving 0.05 c c of diphtheria toxin. The animal survived.

TABLE 6

FINDINGS IN EXPER. 6

	Total Leukocytes	Percentage							
		Amphophile	Basophile	Eosinophile	Lymphocytes	Large Mononuclear	Transitional	Myelocytes	Degenerated Cells
Rabbit A:									
Before inoculation.....	10,900	47.0	5.0	0.5	47.0	0.5	0	0	0
½ hour after...	9,200	49.5	4.0	0	44.0	2.5	0	0	0
1 hour after...	8,725	74.0	3.0	1.0	20.0	2.0	0	0	0
2 hours after..	9,800	52.0	2.5	0.5	42.5	2.5	0	0	15
4 hours after..	12,700	61.0	3.5	0	32.0	3.5	0	0	14
6 hours after..	10,475	59.0	2.0	0	37.0	2.0	0	0	0
24 hours after..	9,225	46.0	3.5	1.0	45.0	4.5	0	0	0
48 hours after..	9,675	47.5	4.0	0	44.0	4.5	0	0	0
72 hours after..	13,675	51.5	3.5	1.5	36.0	7.5	0	0	0
96 hours after..	9,850	46.0	3.0	0.5	48.0	2.5	0	0	0
Rabbit B:									
Before inoculation.....	9,000	37.0	6.5	0.5	56.0	0	0	0	0
½ hour after...	8,725	44.5	3.0	0	52.5	0	0	0	0
1 hour after...	9,250	41.0	4.5	1.5	49.0	4.0	0	0	0
2 hours after..	10,475	51.0	6.5	0.5	34.0	8.0	0	0	0
4 hours after..	12,875	48.0	4.0	2.0	37.0	9.0	0	0	0
6 hours after..	11,925	39.0	4.0	0.5	49.5	7.0	0	0	0
24 hours after..	10,425	40.0	4.0	1.5	47.5	7.0	0	0	0
48 hours after..	12,225	52.0	4.0	0.5	35.0	8.5	0	0	0
72 hours after..	9,875	42.0	3.0	0	46.0	9.0	0	0	0
96 hours after..	9,650	46.5	4.5	0	43.0	6.0	0	0	0

Experiment 7.—The leukocytic exudate was suspended in various amounts of diphtheria toxin incubated at 37 C. to test its injurious effect and also to see whether this effect can be neutralized by the antitoxin. Smears were made every half hour and stained with methylene blue. The results are indicated in table 7.

TABLE 7

FINDINGS IN EXPER. 7

Leukocytes	Toxin	Salt Solution	Results		
			½ Hour	1 Hour	1½ Hours
0.25 c c	0.1 c c	0.65 c c	+	+	+
0.25 c c	0.075 c c	0.675 c c	+	+	+
0.25 c c	0.05 c c	0.7 c c	0	+	+
0.25 c c	0.025 c c	0.725 c c	0	+	+
0.25 c c	0.01 c c	0.74 c c	0	+	+
0.25 c c	0.0075 c c	0.7425 c c	0	0	±
0.25 c c	0	0.75 c c	0	0	0

TABLE 7.—Continued

Leukocytes	Toxin	Antitoxin	Salt Solution	Results		
				½ Hour	1 Hour	1½ Hours
0.25 c c	0.1 c c	0.1 c c	0.55 c c	+	+	+
0.25 c c	0.075 c c	0.1 c c	0.575 c c	+	+	+
0.25 c c	0.05 c c	0.1 c c	0.6 c c	0	0	±
0.25 c c	0.025 c c	0.1 c c	0.625 c c	0	0	0
0.25 c c	0.01 c c	0.1 c c	0.64 c c	0	0	0
0.25 c c	0.0075 c c	0.1 c c	0.6425 c c	0	0	0
0.25 c c	0	0.1 c c	0.65 c c	0	0	0

+, a definite degeneration of leukocytes; ±, doubtful; 0, negative.

It is evident that the amount of diphtheria toxin is an important factor in determining the leukocytic reaction. A massive dose as a rule leads to leukopenia, which prevails throughout the course of the intoxication. The blood smears now show marked retrogressive changes in the leukocytes, and the marrow after death presents no evidence of hyperplasia, the marrow cells being more or less degenerated. It seems safe to say that this leukopenia is due to the directly injurious effect of diphtheria toxin on the marrow and the leukocytes in the circulation. It has been thought that when leukopenia occurs after an intravenous injection it may be brought about by an irregular distribution of the leukocytes in internal organs. In my work the organs taken from the animals in the stage of leukopenia in certain cases presented some evidence of accumulation of leukocytes and in others no difference in comparison with control specimens so far as concerns the number of leukocytes. In immunized animals 0.05 c c of diphtheria toxin produced no appreciable decrease in the number of leukocytes, whereas in the nonimmunized animals the same quantity of toxin caused leukopenia about 6 hours after inoculation. This difference indicates that the antitoxin is able to neutralize the destructive effect of diphtheria toxin on the leukocytes. In experiment 4 there was a progressive leukocytosis with a fatal termination. This seems to be in accord with the results of Gabritchewsky and others but, on the other hand, it must be understood that the amount of toxin used in this experiment was comparatively small and such an amount of toxin may be detrimental to the life of the animal and yet not so destructive to the marrow and the leukocytes in the circulation that leukopenia arises. Myelocytes were found in all the rabbits injected with toxin. In experiment 2 the number of myelocytes was so large that a differential count would remind one of an acute myeloid leukemia, but the total number of leukocytes and the depletion of marrow rather speak

against such deduction. It is difficult to account for the entry of normal myelocytes into the circulation when the marrow is undergoing marked degeneration, but in almost all cases I found a few cells that still maintained their integrity, and these cells, which I believe escaped the toxic effect, subsequently would migrate into the circulation to meet the demand.

The diphtheria toxin acts on both the nucleus and cytoplasm of all the types of leukocytes, and the degree of the leukocytic degeneration largely depends on the quantity of toxin. Furthermore, antitoxin apparently is able to check this injurious effect as illustrated by experiments 6 and 7. In severe cases the nuclei of amphophiles were swollen and pale or became excessively faint. Sometimes they increased in lobulation and stained well with basic dyes. The pyknosis was encountered rarely. The granules of amphophiles were swollen and few in number, and scattered on a pale pink cytoplasm. The nuclei of lymphocytes usually appeared normal, but in acute cases they also underwent the same changes as those of amphophiles. Their cytoplasm was occasionally fragmented or converted into dark spherical bodies 3-4 in number attached to the nucleus. The so-called basket-shaped cells were seen at times.

The changes in the marrow vary with the amount of diphtheria toxin and somewhat also with the duration of action. In addition to the hyperplasia which has been regarded as a usual occurrence in all acute infections, there were various degenerative changes which occurred in a marked degree in the animals inoculated with a massive dose of diphtheria toxin. The diphtheria toxin chiefly affected the nuclei, but the cytoplasm was also more or less involved. The nuclei of myelocytes usually appeared to be swollen, and the chromatin became aggregated into spherical bodies around the perinuclear membrane and stained strongly with basic dyes. This condition probably indicates an early stage of karyorrhexis. Occasionally these spherical bodies were seen in groups here and there in the areas of degeneration. The cytoplasm at first appeared granular and stained well with eosin; later it became swollen and vacuolated and also its affinity for eosin seemed to be lost. The marrow as a rule underwent a hyperplasia in the early stages of experiments regardless of the amount of diphtheria toxin. In experiment 4 one of the animals showed a hyperplasia of small cells with small round and deeply staining nuclei. Of these cells some possessed a large quantity of cytoplasm with an uneven edge,

some had a narrow pink rim around the nucleus and still others presented only a deeply staining nucleus. By the smear method it was found that the majority of the cells were of the lymphoid type, as described by Councilmann, Mallory and Pearce. At this point it is worthy of mention that this animal had lymphocytosis shortly before the death. Megalokaryocytes were few in number and all more or less degenerated, but in the immunized animals they were frequently encountered. The occurrence of phagocytes seemed to have no relation to the stage of inoculation, which is contrary to Dickson's observation, as he found them more numerous in the early stages. Hemorrhage and congestion were of frequent occurrence.

The constant change in the spleen was karyorrhexis, usually in the malpighian bodies. The nuclear fragments in most cases were scattered throughout the lesion and sometimes contained in phagocytes which had a large and pale nucleus and a pink granular cytoplasm with an irregular edge. Occasionally the chromatin of lymphocytes in the malpighian bodies was seen as dark spherical bodies around the perinuclear membrane. The nuclei of the lymphocytes in the splenic pulp and of the reticular cells in the early stage were shrunken and dark, and they appeared to be similar to polymorphonuclear leukocytes. This is probably an early stage of degeneration prior to karyorrhexis. Around these cells dark spherical bodies of various size were encountered also. The so-called epithelioid cells usually occurred in the spleen taken from the immunized animals or from the animals killed in the early stage. These cells were usually definitely marked out and arranged in rows and occasionally scattered among the lymphocytes. The nuclei were large, pale and oval or round, occasionally with a nucleolus which stained pink, and the cytoplasm stained poorly with eosin. None of these cells presented any evidence of phagocytic activity. I assume that they were lymphocytes and reticular cells which were undergoing a retrogressive change. The congestion and hemorrhage occurred nearly in all cases but the degree of the latter varied with the severity of infection. Hyaline degeneration was demonstrated in the mild cases.

The changes in the lymph nodes were practically the same as those in the spleen and for this reason I shall deal with only the conditions which were not found in the latter. In experiment 5 the sinuses of the lymph nodes were distended either with yellowish granular coagulum or with phagocytes and erythrocytes. In this case the lymph node also showed a certain degree of hyperplasia and degeneration.

The observations on the effect of diphtheria toxin on the erythrocytes revealed nothing important. In one case there was a slight decrease in the number of erythrocytes and the percentage of hemoglobin with an occasional appearance of normoblasts and crenated erythrocytes.

CONCLUSIONS

Diphtheria toxin is destructive to the leukocytes in vivo as well as in vitro.

In massive doses it usually produces a retrogressive change in the hemopoietic organs.

Leukopenia in animals inoculated with a massive dose of diphtheria toxin is in all probability due to the degeneration of the hemopoietic organs and the leukocytes in the circulation caused by diphtheria toxin.

The so-called epithelioid cells in the malpighian bodies seem to be the degenerated lymphocytes and reticular cells.

Antitoxin in a proper portion is able to neutralize the destructive effect of diphtheria toxin on the hemopoietic organs and on the leukocytes in vivo and vitro.

Plate 1

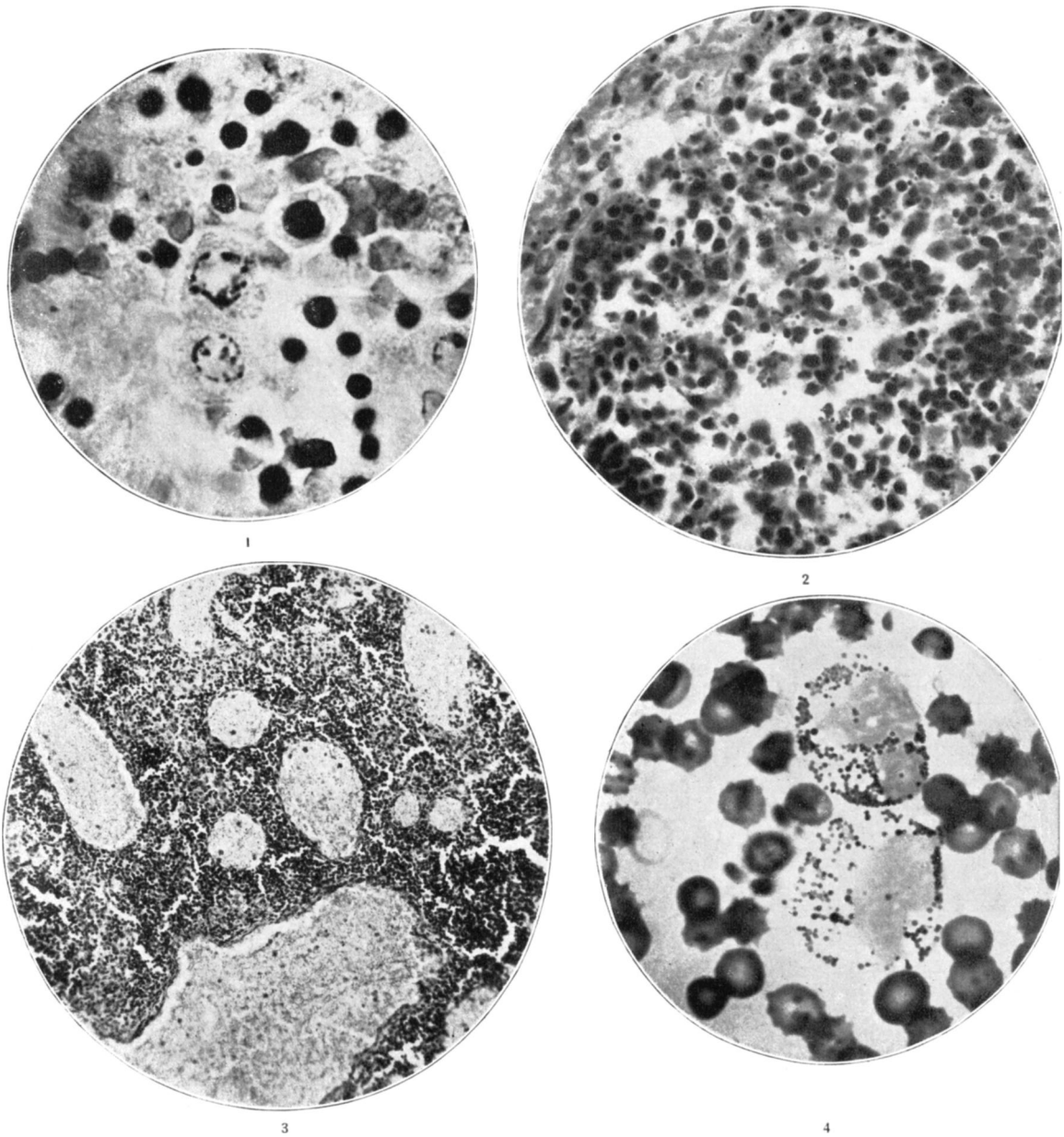

Fig. 1 (Exper. 2).—Marrow showing an early stage of karyorrhexis of myelocytes, and also a large number of cells with round and deeply staining nuclei; slightly reduced from a photomicrograph. X 1200.

Fig. 2 (Exper. 4).—Karyorrhexis in malpighian body; slightly reduced from a photomicrograph. X 600.

Fig. 3 (Exper. 5).—Lymph node showing karyorrhexis, hyperplasia and a marked distention of sinuses; slightly reduced from a photomicrograph. X 170.

Fig. 4 (Exper. 3).—A blood smear about 2 hours before the death of the animal showing karolysis of myelocytes. To their left a degenerated lymphocyte with 3 dark spherical bodies; slightly reduced from a photomicrograph. X 1200.